

Migratory patterns of cloned neural crest melanocytes injected into host chicken embryos

(migration/pigment cells/injection technique/differentiation)

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ABSTRACT Cloned quail melanocytes grown in tissue culture for 8 days or more were injected into 2½-day-old chicken embryos. The pigment cells were placed directly into the somitic lumen by means of an injection micropipette. This technique for introducing marked neural crest cells into host embryos causes far less damage than previous methods which require extirpation and replacement of the neural tube. In addition, small numbers of homogeneous cells can be implanted by this procedure. When injected into one of the posterior somites, cultured pigment cells migrated along the ventral neural crest pathway. Three days after injection the melanocytes had migrated ventral to the dorsal root ganglia and prevertebral and primary sympathetic chain ganglia and were seen associated with the adrenal gland and aortic plexi. Melanocytes were frequently found in or adjacent to the gonads and often had migrated as far as the gut.

In studies of cell differentiation and migration, the neural crest has been a system of particular interest. This temporary embryonic structure originates on the dorsal side of the closing neural folds; its cells subsequently migrate along well-defined pathways in the vertebrate embryo. In the trunk region, the crest gives rise to several derivatives, including spinal ganglia, sympathetic ganglia, Schwann cells, and melanocytes. Differentiation of these cells has been studied in previous reports by replacing host neural crest with crest cells identified by radioisotopic or biological markers (1-3). The objective of this study is to describe a technique for introducing small numbers of homogeneous crest cells back into the embryo without inflicting the damage associated with previous microsurgical approaches to this problem.

The routes of neural crest migration have been well documented in the avian embryo (1-4). After closure of the neural tube in the trunk region, the crest cells emigrate from the dorsal aspect of the tube in two distinct streams: a dorsolateral stream, which migrates just under the ectoderm and eventually differentiates into melanocytes, and a ventral stream of cells, which migrates between the neural tube and the somites. The cells choosing the ventral pathway differentiate into sensory and sympathetic neurons, adrenomedullary cells, and supporting cells. These crest cells localize in three main areas (Fig. 1): some cells settle in a dorsal position, between the neural tube and the somites and form sensory ganglia; others migrate lateral to the notochord and give rise to the primary sympathetic chain; and some cells migrate still further ventral or lateral (or both) to the dorsal aorta and differentiate into the adrenal medulla, aortic plexi, and some cells of the metanephric mesenchyme (5).

Previous methods for studying the migration and localization of neural crest cells *in situ* subjected the embryo to severe trauma. In the chicken embryo, a length of the neural tube and

sometimes notochord was extirpated and replaced with one whose cells were suitably marked so that the migrating crest cells could be identified (1-3). Onset of neural crest migration is usually delayed several hours after such a transplant is made (1). To circumvent this problem and other possible traumatic consequences of neural tube transplantation, we introduced crest cells back into the embryo by means of an injection method. The behavior of cloned melanocytes returned to the early embryo by the injection technique is described here.

MATERIALS AND METHODS

Preparation of Cells. Primary cultures of neural crest cells were prepared from neural tubes of Japanese quail (*Coturnix coturnix japonica*) embryos as described (6). Embryos were incubated for 47 hr at 38°C, at which time their developmental age was comparable to chicken stages 14-15 (7). Neural tubes were isolated from surrounding tissue by brief trypsinization. Five to eight neural tubes were then placed on collagen-coated petri dishes containing culture medium (Eagle's minimum essential medium, horse serum, embryo extract, and gentamycin). Crest cells began migrating away from the neural tubes after 2-3 hr. After 24 hr the neural tubes were carefully scraped away, the growth medium was changed, and the cultures were allowed to grow for another 24 hr. For those experiments in which somite cultures were used, somites were isolated from neural tube and surrounding tissue by brief trypsinization and plated on collagen-coated petri dishes. Cultures were allowed to proliferate for 24-72 hr before injection (see below for dissociation and injection techniques).

Preparation of Neural Crest Secondary Cultures. Forty-eight hours after the primary cultures were established, the neural crest cells were dissociated by washing cultures twice with calcium- and magnesium-free phosphate-buffered saline (NaCl/P_i) and then incubating at 37°C for 3 min in 0.25% trypsin in NaCl/P_i. The reaction was stopped by rinsing with culture medium containing endogenous trypsin inhibitors. Cells were suspended by gentle pipetting and counted in a Petroff-Hausser bacterial cell counter. Clonal cultures were inoculated with 25-50 cells into 60-mm Falcon petri plates coated with a thin layer of gelatin. Cultures were fed 5 ml of fresh medium every 3 days.

Clones of neural crest cells were allowed to proliferate for 8 days or longer. By this time a majority of clones was pigmented (6). These were encircled with porcelain penicylinders stuck to the petri plates with sterile stopcock grease. These cloned cells were removed by washing twice with NaCl/P_i, followed by mild trypsinization in NaCl/P_i. After dissociation, the cells were pelleted and then resuspended in approximately 5 µl of medium. In later experiments, cells were removed by

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Abbreviation: NaCl/P_i, calcium- and magnesium-free phosphate-buffered saline.

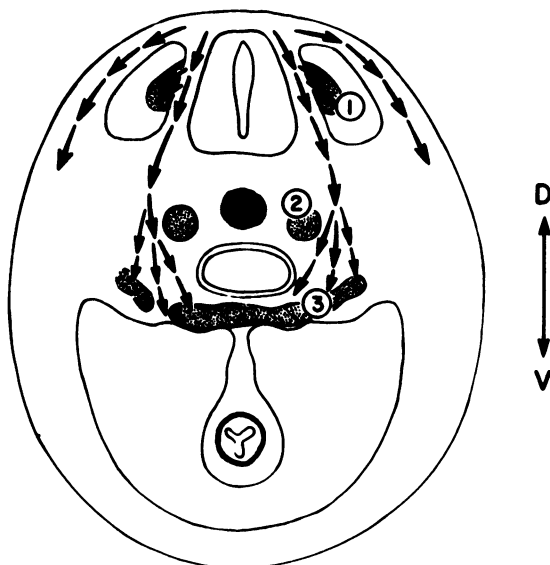


FIG. 1. Diagram illustrating the normal routes of neural crest migration in the trunk region of the avian embryo. Cells choosing the ventral pathway localize in three main areas: 1, the sensory ganglia; 2, the primary sympathetic chain; and 3, the adrenal gland, aortic plexi, and some cells of the metanephric mesenchyme. Crest cells following the dorsolateral pathway migrate under the ectoderm and become skin melanocytes.

washing with NaCl/P_i alone and gently pipetting. Migration of the pigment cells injected back into the embryo was not influenced by the method of secondary dissociation.

Injection Techniques. The cell suspension was back-filled into fine glass micropipettes with openings of approximately 50 μ m. These were prepared from glass capillary tubing with drawn-out, beveled tips. After filling, micropipettes were mounted on an electrode carrier and connected to a forced-air injection apparatus. The cell suspension was then injected into the desired location of the embryo. White Leghorn chicken embryos, incubated at 38°C until stage 14–17 (7), were used as hosts in all experiments. Embryos were fixed for histologic examination in Zenker's solution and subsequently embedded in paraffin. Serial sections were cut at 10 μ m and stained by the Feulgen–Rossenbeck method for DNA. This method permits us to distinguish between quail cells and those of the chicken based on nucleolar chromatin patterns seen in the interphase nucleus (2).

RESULTS

Pigmented cells were injected as marker cells into several sites in the trunk in order to determine the feasibility of this technique for introducing small numbers of cells into the embryo. Four injection sites were tested: the space between the neural tube and somites, the lumen of the neural tube, the cavity of the somite, and the intermediate mesoderm. The space between the neural tube and somites was tested first because crest cells normally move through this area while migrating ventrally. When injected into this area, pigmented cells could not be found in histological sections in any of the four embryos examined immediately after injection. Possibly, the force of the injection dispersed the cells. This seems likely since the embryo in this region is very loosely organized. The cavities of the neural tube or somites were tested next as injection sites to circumvent dispersion of the cells. The neural tube was not satisfactory since upon injection it usually ruptured along its dorsal surface, releasing the newly introduced melanocytes. In the two cases where melanocytes were retained, they failed to migrate out

of the lumen. The somites, however, proved a suitable site to prevent scattering of the injected cells. The seven embryos sectioned immediately after injection showed pigment cells only within the somitic lumen (Fig. 2a) or, infrequently, an additional few cells were seen dorsally in the needle track. In no instances were cells seen ventral or lateral to the somites just after injection. When cells were injected lateral to the somites in the intermediate mesoderm, in none of the seven cases could pigment cells be found. Again, the cells seemed to be forced from the site as a consequence of the injection itself.

The somites were therefore chosen as a site for the injection of quail neural crest cells; in particular, the posterior somites offered several advantages. At the time of injection they are epithelial sacs with a cavity suitable for initially retaining the injected cells. In the course of normal somitic development the cells of the ventromedial wall of the somitic epithelium disperse, exposing the injected pigment cells to the ventral pathway of host neural crest cell migration. In the embryos considered in the following experiments, cloned pigment cells were injected into one of the last six somites (Fig. 2a). In the majority of instances, however, cells were injected into the second or third to last somite. At this level, host crest cell migration had not yet begun. Normally, crest migration occurs as a wave which starts at the anterior of the embryo and moves progressively posteriorly. The posterior limit of migrating crest cells is usually one to three segments from the most recently formed somites (8) in the stages used here. Therefore, the injected clones of melanocytes were released from the somites and exposed to the ventral pathway at approximately the same time as the endogenous neural crest cells were starting to leave the neural tube.

Damage to the host embryo, and somite in particular, was minor when the injection was performed properly. The injection micropipette, with an opening of about 50 μ m, pierced the ectoderm and underlying somite. The melanocytes and a small volume of fluid were released from the needle with a quick pulse of forced air, causing the somite to expand slightly and subsequently to return to its previous size. After withdrawal of the micropipette, a small puncture wound remained in the roof of the somite and overlying ectoderm which healed quickly. The number of injected cells varied from a few to several hundred per somite. Care was taken not to lower the needle too far, to prevent passing through the somite and possibly damaging the subjacent dorsal aorta.

The behavior of pigmented quail cells injected into chicken embryos demonstrates the feasibility of this technique for reintroducing small numbers of cloned cells into the embryonic environment. Pigment clones grown for 8, 11, 15, and 24 days *in vitro* survived and migrated in chicken embryos. A few hours after injection, the rounded melanocytes began extending processes, demonstrating their viability and suggesting the initiation of migration. The possibility of confusing injected and host melanocytes was ruled out since the cloned melanocytes were introduced 5 days before host melanogenesis. Furthermore, the experiments were terminated before the beginning of host melanin production and host melanocytes die shortly after they form, the consequence of a lethal mutation affecting these cells (9). The injected clones of melanocytes migrated along a route corresponding to the ventral route followed by endogenous neural crest cells in the avian embryo (see Fig. 1). The location of quail cells was examined at various times after injection in serially sectioned embryos. The duration of time spent in clonal culture did not affect the route or the time course of migration, so data from different ages of injected clones were considered collectively (Table 1). The cells were injected into somites of 2½-day-old hosts, at which time there was little or

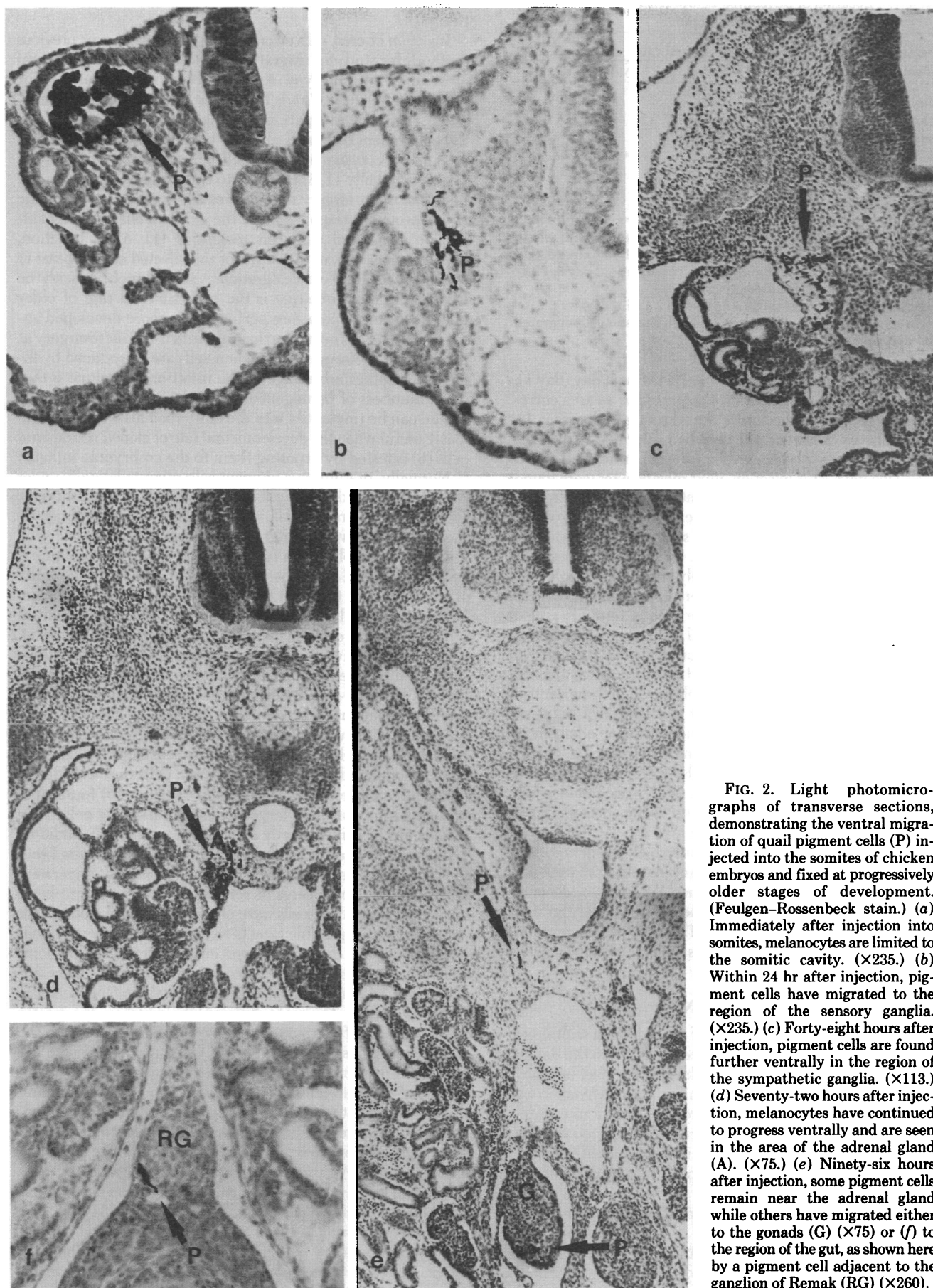


FIG. 2. Light photomicrographs of transverse sections, demonstrating the ventral migration of quail pigment cells (P) injected into the somites of chicken embryos and fixed at progressively older stages of development. (Feulgen-Rossenbeck stain.) (a) Immediately after injection into somites, melanocytes are limited to the somitic cavity. ($\times 235$.) (b) Within 24 hr after injection, pigment cells have migrated to the region of the sensory ganglia. ($\times 235$.) (c) Forty-eight hours after injection, pigment cells are found further ventrally in the region of the sympathetic ganglia. ($\times 113$.) (d) Seventy-two hours after injection, melanocytes have continued to progress ventrally and are seen in the area of the adrenal gland (A). ($\times 75$.) (e) Ninety-six hours after injection, some pigment cells remain near the adrenal gland while others have migrated either to the gonads (G) ($\times 75$) or (f) to the region of the gut, as shown here by a pigment cell adjacent to the ganglion of Remak (RG) ($\times 260$).

Table 1. Location of pigmented neural crest cells at various times after injection

Location of cells	No. of embryos			
	Day 1	Day 2	Day 3	Day 4
Area 1	4			
Area 2	2	2		
Area 3	6	8	6	3†
Area 4			3*	3
Total embryos	12	10	9	6

Area 1, sensory ganglia; area 2, primary sympathetic chain; area 3, adrenal gland, preaortic plexi, metanephric mesenchyme; area 4, gut. Day 1, 24 hr after injection; day 2, 48 hr after injection; day 3, 72 hr after injection; day 4, 96 hr after injection.

* One embryo has pigment cells in both area 3 and area 4.

† One embryo had a single cell in area 2 though its sister cells migrated more ventrally.

no indication of donor cell migration. By the next day (day 1), the pigment cells in a few embryos migrated to an area corresponding to the site of the future dorsal root ganglia (area 1). In most embryos, however, the cells had migrated further, to the site of the sympathetic ganglia (area 2) or adrenal gland (area 3). Two days after injection, most melanocytes were found only in area 3, although a few pigment cells were seen in area 2. Three days after injection, melanocytes in all embryos had migrated at least as far as the ventral surface of the aorta, the site of the sympathetic aortic plexus. In some instances cells migrated as far as the gut. Generally, however, implanted melanocytes were seen in regions where endogenous crest cells normally localized. Melanocytes were usually found in area 3 located below and lateral to the dorsal aorta. They also settled in regions that gave rise to the preaortic plexus, the cortical body, and suprarenal gland. It should be emphasized that on day 0, no injected melanocytes were observed in these ventral positions. By the fourth day, a larger percentage of pigment cells had reached the gut (Table 1) and were seen adjacent to the visceral musculature, the ganglion of Remak (Fig. 2f), or the dorsal mesentery. Pigment cells also were frequently seen both adjacent to and within the gonads. In only one instance was an injected pigment cell seen still dorsal to the aorta by day 4 though its sister cells had migrated to area 3.

In order to examine the migratory pattern of non-neural crest cells, somite cells were injected in combination with melanocytes into chick hosts. In five of five cases the quail somite cells remained in a dorsal position while the melanocytes migrated ventrally as in previous experiments. This supports the premise that the ventral migratory behavior is a phenomenon specific to neural crest cells.

DISCUSSION

The results demonstrate the utility of the injection technique for introducing cultured quail crest cells back into the developing chicken embryo. Not only do the cells survive, but they also migrate ventrally along the pathway normally taken by precursors to the sensory, sympathetic, and Schwann cell derivatives. The cultured melanocytes originally injected into the somites move progressively ventrally until they localize in the area of the adrenal medulla/aortic plexi or enter the dorsal mesentery and migrate to the gut.

Quail cells can be distinguished from chicken cells when stained by the Feulgen-Rossenbeck method because of the condensed heterochromatin unique to the quail cells (2). In embryos injected with cloned melanocytes, no unpigmented quail cells were identified. This indicates that none of the me-

lanocytes lost or altered their phenotype during the migration.

Injection of crest cells offers several advantages over previous methods for studying migration and localization of the neural crest in living embryos. First, injection causes only minor damage to the host. This is in contrast to transplantation techniques involving extirpation of a length of host neural tube and its replacement by a labeled donor tube. The implanted neural tube and accompanying crest cells are marked biologically or radioisotopically (1-3) in order to distinguish them from the host. The microsurgery required for this operation is traumatic and is probably responsible for the delay in onset of crest migration associated with this technique (1). After injection, however, the onset of migration of the injected cells appears to coincide with local crest migration. A second problem with the transplantation technique is the poor survival rate of older embryos and of operations performed in more developed anterior levels (8). The hemorrhaging caused by microsurgery at these stages is circumvented when cells are introduced by injection. Another advantage of the injection technique is that small numbers of homogeneous cells derived clonally or otherwise can be implanted into the embryo. This will be particularly useful when the developmental fate of cloned neural crest cells (6) is tested by exposing them to the embryonic milieu.

Normally, presumptive pigment cells migrate laterally under the ectoderm, and one might expect that mature pigment cells, should they migrate at all, would do the same. The injected melanocytes, however, migrate ventrally rather than laterally. One possible explanation for this is that the injected pigment cells are released from the ventromedial aspect of the somite at a site adjoining the ventral pathway of neural crest migration. Therefore, initial orientation of the cells is onto the ventral route; this might exclude them from the dorsolateral pathway, which presumptive melanocytes normally follow. This idea is supported by preliminary observations that frank skin melanocytes from 11-day quail embryos also follow the ventral pathway when introduced into the somites. An alternative explanation for the ventral direction taken by the cultured, neural crest-derived melanocytes is that these cells might in fact represent modulated neuronal or Schwann cells that would normally migrate ventrally. Cowell and Weston (10) found that sensory ganglia produce large numbers of pigment cells if the ganglia are explanted shortly after their formation. In addition, Nichols and Weston (11, 12) observed a population of small cells they thought to be precursors of supportive cells that underwent melanogenesis in cultures of sensory ganglia and peripheral nerves. Whether these cells were pluripotent neural crest or cells restricted to two possible fates (i.e., to become supportive cells or pigment cells, depending upon environmental interactions) was not clear. Environmental conditions similar to those encountered in our cultures influenced melanogenesis in the foregoing studies. The more dispersed the ganglion, the greater the percentage of pigmented cells; permissive medium and the nature of the substratum also affected melanin synthesis. In our clonal cultures, neural crest cells were grown under highly dispersed conditions and fed with medium containing many factors common to those considered "permissive" for melanogenesis. Consequently, injected melanocytes might be derived from small supportive cell precursors similar to those described by Nichols and Weston. This could explain migration of these cells along the ventral pathway as opposed to the dorsolateral pathway followed by presumptive melanocytes.

The extensive migration of crest cells in the embryo reflects the natural migratory tendency of these cells and the ability of the environment to encourage migration. That the environment promotes migration is supported by our results. Pigmented cells

that are not motile *in vitro*, as shown by time-lapse cinematography (unpublished observation) and direct observation (13), migrate enthusiastically when injected back into the embryo and exposed to the ventral crest pathway. LeDouarin *et al.* (14) also observed a similar onset of migratory behavior from previously nonmotile crest derivatives when they implanted parasympathetic ganglia alongside the neural tube at the time of ongoing host neural crest migration. The ganglionic cells, even though beginning to acquire cholinergic traits, migrated ventrally and localized in ortho- and parasympathetic sites normally populated by crest cells. Whether the migrating cells were in fact differentiating cholinergic neuroblasts or a population of pluripotent stem cells has not been resolved. The migratory behavior of both the pigment cells and ganglion cells suggests that the ventral route influences initiation, direction, and localization of not only immature crest cells, but also their derivatives.

Little is known about mechanisms regulating distribution and ultimate localization of migrating neural crest cells. Certainly the environment plays a critical role in distribution of the cells. Noden (15) demonstrated that the unique patterns of crest distribution in the head region of avian embryos are largely directed by environmental factors. Patterning of dorsal root ganglia in Amphibia is conditional upon previous patterning of the somites (16). LeDouarin *et al.* (17) have shown that crest from midtrunk levels will migrate as crest from vagal levels when implanted in that area. In other words, their distribution is influenced by the environment in which they migrate. We know less about what affects final localization of crest cells. Cessation of migration might be due to intrinsic changes in the crest cells themselves, alterations in the migratory pathway, or a combination of the two. Brauer (18) suggested that physical obstructions in the pathway, such as large blood vessels or nerves, influenced localization of the migrating cells. Surprisingly, the injected melanocytes were found associated with presumptive ganglioblasts of the aortic plexi and adrenal medullary cells. In addition, some of the melanocytes continued into the dorsal mesentery and eventually populated the gonads, kidney, and intestine. The orthosympathetic and enteric sites normally are not populated by pigment cells in the quail (donor) or White Leghorn (host) although the enteric sites are pigmented in several strains of pigmented chickens (19). The

nonrandom distribution of the injected pigment cells suggests they respond to environmental cues that direct the localization of undifferentiated crest cells. The extent of ventral migration by the injected cells is also noteworthy. Crest cells from midtrunk levels normally migrate only as far ventrally as the orthosympathetic para-aortic ganglia, certainly not into the mesenteries or beyond (20). When vagal neural crest cells are implanted in place of midtrunk crest cells, however, they migrate as far ventrally as the gut and contribute to the enteric ganglia but do not differentiate into pigment cells. The extensive migration of injected melanocytes into the gut might represent trophic influences or, alternatively, modifications of the cell surface permitting migration beyond that normally seen for midtrunk crest cells.

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